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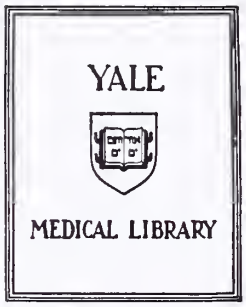
Na^+ AND Cl^- FLUXES, AND EFFECTS OF PHARMACOLOGICAL AGENTS ON THE SHORT-CIRCUIT CURRENT OF THE ISOLATED RABBIT IRIS-CILIARY BODY

by

SAMUEL RICHARD PESIN

1985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Na^+ and Cl^- Fluxes, and Effects of Pharmacological Agents on the Short-Circuit Current of the Isolated Rabbit Iris-Ciliary Body

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

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ABSTRACT

Na^+ AND Cl^- FLUXES, AND EFFECTS OF PHARMACOLOGICAL AGENTS ON THE SHORT-CIRCUIT CURRENT OF THE ISOLATED RABBIT IRIS-CILIARY BODY

Samuel Richard Pesin

1985

Aqueous humor formation is partly due to ultrafiltration and largely due to secretion. Investigations of transport properties in isolated iris-ciliary body preparations provide a clearer understanding of ciliary epithelial secretion and thereby aqueous humor production. In vitro experimentation allows the study of isolated ciliary epithelial functions independent of neurologic, circulatory, and other systemic effects. Also, the direct effect of pharmacological agents on the ciliary epithelium can be studied.

Analyses of tissue ionic requirements, ion fluxes, and pharmacologic effects will add to our present knowledge of ciliary epithelial active transport properties. By using modified Ussing-Zerahn chambers, within which are mounted isolated iris-ciliary body membrane preparations, one can measure transepithelial electrical potential difference, short-circuit current, and ion fluxes under normal and chemically or pharmacologically altered states. These investigations can better elucidate the mechanisms responsible for ciliary epithelial ion transport, as well as the role this epithelium plays in aqueous humor formation. Thus, an overview of active ion transport processes present in this preparation can be obtained. Furthermore, several of the electrophysiologic contradictions of previous studies on rabbit iris-ciliary body can be resolved.

Na^+ and Cl^- Fluxes, and Effects of Pharmacological Agents on the Short-Circuit Current of the Isolated Rabbit Iris-Ciliary Body

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I. INTRODUCTION

A. Clinical Background

Glaucoma is a common and serious eye disease affecting the vision of about 1.2 million people in the United States, and causing visual impairment in over 200,000, of whom 62,000 are legally blind. About 180,000 new cases are diagnosed every year.[1] The great majority of glaucoma patients are treated with pharmacological agents while some require surgery. Although it has become apparent that there are 30 to 40 different types of glaucoma with different underlying causes, one of the most common characteristics is an intraocular pressure (IOP) above that of normal eyes.

Since the eye is an elastic globe containing a gel (vitreous body) posteriorly and aqueous humor fluid secreted and slowly circulating anteriorly, the IOP and sphericalness of the eye are maintained by the secretion-outflow system of the aqueous humor. If the normal outflow is obstructed, a buildup of pressure within the eye results. This can lead to optic nerve damage with the resultant glaucoma manifested as either visual field loss (functional damage) or optic nerve cupping (tissue damage).

B. Aqueous Humor Circulation

Aqueous humor is produced by the ciliary body epithelium and is liberated into the posterior chamber of the eye (Figure I). From here, it flows through the pupil, into the anterior chamber, and returns to the bloodstream. As in any hydrodynamic

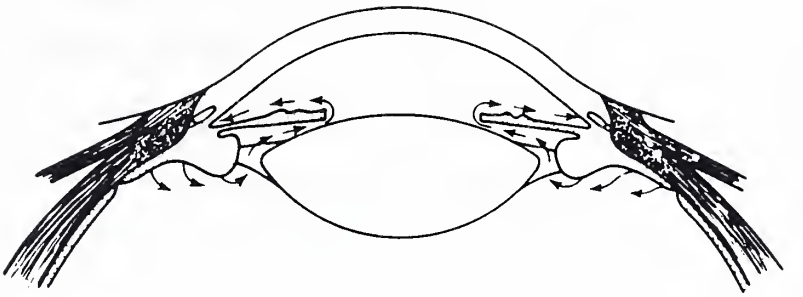

Figure I. Diagram of aqueous humor circulation after secretion through posterior chamber, pupil, and anterior chamber and its outflow via the canal of Schlemm. (From ZADUNAISKY, 1978)

system, the rate of flow of aqueous humor depends on the rates of aqueous formation and outflow, which in turn partially depends on pressure gradients and equivalent resistance coefficients of inflow and outflow channels. In the steady state, when the IOP is neither rising nor falling, the rate of aqueous formation equals that of the outflow. Any restriction to outflow results in a new steady state with a higher IOP. Conversely, a decrease in the rate of inflow will change the steady state condition to one with a lower IOP.

C. Aqueous Humor Dynamics

IOP is a dynamic measurement subject to a wide variety of ocular and nonocular influences. Aqueous humor dynamics involve (1) the composition of the aqueous humor and its rate of formation by ciliary epithelial active secretion, ultrafiltration (dialysis in the presence of hydrostatic pressure), the active transport of materials out of the eye, and diffusional exchange between both the vitreous and blood; (2) outflow of aqueous humor from the anterior chamber by the conventional pressure-sensitive flow through the trabecular

meshwork into the canal of Schlemm and by the unconventional pressure-insensitive uveoscleral flow; and (3) episcleral venous pressure (recipient veins). These aforementioned relationships can be expressed under steady state conditions (when the IOP is neither rising nor falling) by the following equation:[2]

$$\text{aqueous formation rate} = \frac{\text{IOP - episcleral venous pressure}}{\text{trabecular resistance}} + \text{uveoscleral flow}$$

D. Structure of the Ciliary Epithelium

The ciliary body, located between the iris and the choroid, consists of the ciliary muscle and ciliary processes. (Figure II)

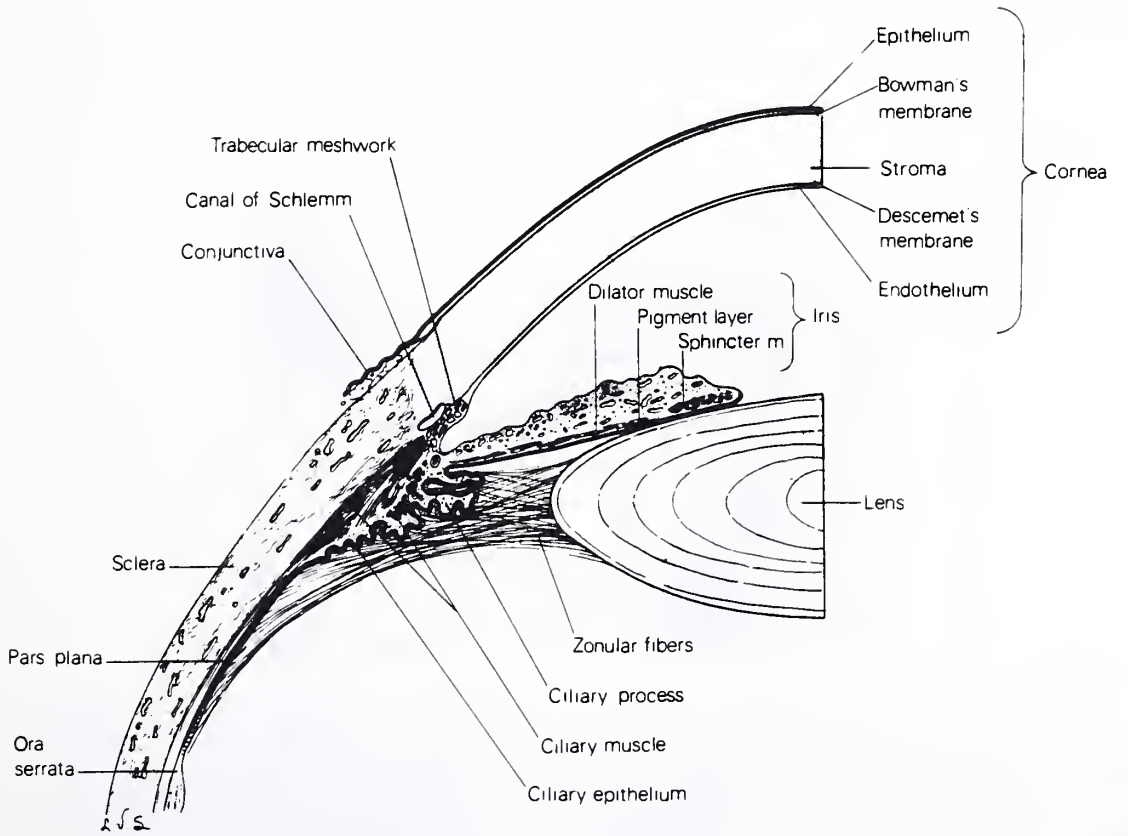

Figure II. Anterior chamber angle and surrounding structures. (From VAUGHAN and ASBURY, 1983)

These processes, which number about 70, are triangular projections at the root of the iris that extend inward from the ciliary body as radiating villiform ridges. The group of processes are essentially floating free in the aqueous humor of the posterior chamber and can be thought of as an everted gland. Each process is composed of blood vessels embedded in a loose connective tissue stroma with a double layer of epithelial cells--the outer pigmented and the inner non-pigmented --lining the inner surface. Although the ciliary body as observed posterior to the iris is a 0.75 cm^2 annulus, the true total surface area of the ciliary epithelium is about 5 - 6 cm^2 in the rabbit because of the extensive convolutions in the tissue. [3,4] The two epithelial cell layers are oriented such that the apices of one cell layer face the apices of the other. (Figure III)

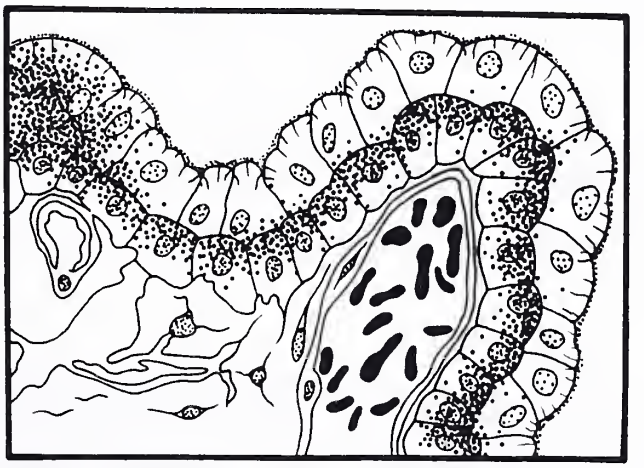

Figure III. Section through ciliary epithelium showing its two layers of cells, pigmented and non-pigmented. On the right side is drawn a blood vessel located within the connective tissue of the stroma. (From ZADUNAISKY, 1978)

The bases of the outer pigmented ciliary epithelial cells face the ciliary body stroma, separated from it by a basal lamina which is a forward continuation of Bruch's membrane. The bases of the inner non-pigmented cells face the posterior chamber (containing the aqueous humor), separated from it by an internal basal lamina which is a forward continuation of the internal limiting membrane of the retina. The adjacent apices of the two epithelial cell layers are elaborately interdigitated and connected by tight junctions representing the blood-aqueous barrier at the level of the ciliary epithelium. Also within the interface between the apices of the two layers are numerous gap junctions suggesting electrical and metabolic coupling between the two layers.[5] It had been previously suggested by Cole[3] that, based on histochemical and anatomical studies, both pigmented and non-pigmented cells participate in the production of aqueous humor. Thus, any cooperation between the two epithelial layers in series necessitates the presence of gap junctions to mediate passage of intracellular ions from the pigmented to the non-pigmented cells.[5]

E. Aqueous Humor Transport Mechanisms

Differences exist between the components of the aqueous humor in the posterior chamber and that in the anterior chamber.[6] This is because the exact composition of the aqueous humor at any location in the anterior or posterior chamber is influenced by uptake and release of metabolites by the surrounding tissues--the lens, iris and cornea. Furthermore, aqueous humor is not just an ultrafiltrate of plasma, since

the concentration of several ions (including sodium, chloride and bicarbonate ions) and ascorbic acid is higher than in plasma. Systems have been demonstrated for inward transport of substances, e.g., ascorbate;[7] or outward transport, e.g., organic and inorganic anions.[8,9] IOP has been shown to decrease following the inhibition of cell-mediated processes, e.g., inhibitors of carbonic anhydrase lower aqueous humor bicarbonate content leading to a drop in aqueous humor production;[10] and ouabain, which inhibits Na^+-K^+ ATPase, reduces sodium influx resulting in a decrease in aqueous humor inflow.[11]

II. INVESTIGATIONS INTO AQUEOUS HUMOR FORMATION

A. In Vivo Studies--Advantages and Drawbacks

IOP may be influenced by several factors, e.g., systemic and ocular blood pressure,[12] ocular blood flow,[13,14] systemic acid-base status,[15] body temperature,[16,17] numerous hormones such as adrenal steroids,[18] and various neurohumeral mechanisms. While most cases of glaucoma involve an increase in IOP due to a restriction in aqueous outflow, the purpose of most non-surgical treatment is to reduce aqueous humor production. Thus, a clear insight into the mechanisms involved in aqueous humor formation is important. Various techniques are available to study such mechanisms. Important ocular physiologic data has been obtained from in vivo analyses of the aqueous humor in both the anterior and posterior chambers[19] or just perfused posterior chamber aqueous.[11] These methods provide a composite overview of aqueous secretion including blood flow alterations, cornea and lens metabolic processes, development of plasma filtrate in the ciliary process stroma, diffusional exchanges and other processes.

The autonomic nervous system innervates the trabecular meshwork and the ciliary and other ocular blood vessels, and may directly or indirectly regulate ciliary epithelial secretion, aqueous outflow, or blood flow. Glaucoma therapy relies heavily on parasympathomimetic agents, adrenergic agonists or antagonists and carbonic anhydrase inhibitors. Furthermore, numerous other agents have been used experimentally *in vivo* to decrease IOP, includiong Na^+-K^+ ATPase inhibitors (ouabain[20] and vanadate[21]), cyclic AMP,[22] and H_1 antihistamines.[23] Whereas the net effect of each of these agents is a decrease in IOP, the potential site of action is uncertain, e.g., ciliary epithelium, trabecular meshwork, ocular blood vessels, or a combination of these sites. Thus, *in vivo* studies are an inadequate method for determining the direct site of action of these agents.

B. *In Vitro* Studies--Advantages and Drawbacks

In depth studies of aqueous humor transport systems are possible with *in vitro* preparations of the isolated iris-ciliary body. Furthermore, with *in vitro* techniques, the isolated ciliary body can be used to perform such studies as flux measurements, determinations of oxidation-reduction potentials, and measurements of uptake and accumulation of substances. The effects of agents added to the bathing solution of an *in vitro* system must necessarily be due to alterations in the membrane and its transport properties. While the results of *in vitro* methods represent secretion processes present

in vitro, *in vitro* studies can also enable one to study the direct effect of pharmacological agents or ion substitution experiments on the ciliary epithelium and its metabolic functions independent of systemic or local metabolic, neurologic, or circulatory changes.

As in the experiments of Ussing and Zerahn,[24] the isolated iris-ciliary body tissue can be mounted as a disc separating two chambers containing oxygenated saline. However, a limited number of attempts to isolate and mount the ciliary body have been made. The anatomic location of the ciliary body, its direct attachment to the root of the iris and the fragility of this membrane are just a few of the hindrances in isolating and mounting the preparation. Tissue damage during preparation or deprivation of the epithelium from its extensive blood supply could result in some loss of activity with reduced rate of fluid transport.

III. RESULTS OF *IN VITRO* ELECTROPHYSIOLOGY STUDIES OF CILIARY EPITHELIAL TRANSPORT

It has been shown that active transport is dependent on oxidative metabolism, the ciliary epithelium having a high rate of glycolysis.[25,26] In 1962, Cole[27] described a mounted preparation of the isolated rabbit iris-ciliary body (I-CB) in an Ussing-type chamber whereby the membrane potential difference (PD) and short-circuit current (SCC) could be measured in an attempt to establish a relationship between the total current and the ion passage across the tissue. His technique involved mounting the I-CB between two chambers and blocking the

pupil aperture with small discs held in place by pressure. In 1973, Green and Pederson[28] modified Cole's technique by blocking the center of the rabbit I-CB with a glued Lucite disc. Results of Green and Pederson[4,28,29] are similar to those of Cole.[27,30,31]

In vitro Ussing-type studies have shown that ciliary body transport is oxygen- and temperature-dependent and inhibitable by both uncoupling agents and Na^+-K^+ ATPase inhibitors.[4,26,30,31] Varied results have been obtained with different experimental animals. Using rabbit and ox I-CB, Cole[27,30,32] found an electrical potential of about 5mV, the epithelial side positive to the stromal side. However, more recent work with the rabbit I-CB by Kishida, et al.[33-35] and Krupin, et al.[26] reported that the epithelial side was about 1mV negative with respect to the stromal blood side. The discrepancy between Cole's PD orientation and these more recent findings has been suggested by Krupin, et al.[26] as possibly being due to Cole's use of Tris-buffered solution containing no bicarbonate. For, both Kishida, et al.[34] and Krupin, et al.[26] have shown that the transepithelial PD of the isolated rabbit I-CB was affected by the bathing solution composition, and that the epithelial side PD is always negative unless the bathing solution contains no bicarbonate. The epithelial (aqueous) surface of the isolated ciliary body has also been found to be electronegative relative to the stromal side in the cat,[36] toad,[37,38] dog, bovine, and human.[39] Besides the presence of active sodium transport across the isolated ciliary body, chloride transport has been

shown to exhibit varying degrees of importance in the ciliary epithelia of the aforementioned animals.[26,33-36,38,39]

Although reported variations in ciliary body electrical potential and active transport processes may reflect differences in aqueous composition, some of these variations may be due to experimental design. The chemical composition of aqueous humor does differ among species of animals. For example, the aqueous humor of the cat has an excess of chloride compared to plasma, whereas rabbit aqueous humor chloride concentration is similar to that of plasma. The ciliary epithelium can be placed within the category of leaky junctions for a transporting epithelium, having a low value for resistance, transport potential difference, and osmolarity ratio(≈ 1).[31] Values for ciliary epithelial resistance have been reported by Cole[31] to be about $60\,\Omega \cdot cm^2$ and recently by Krupin, et al.,[26] to be about $152\,\Omega \cdot cm^2$.

IV. THESIS PROPOSAL

A. Specific Goals

The goals of this thesis project are to (1) better elucidate the mechanisms involved in formation of aqueous humor, (2) study how pharmacological agents can modify its production, and (3) attempt to resolve some of the contradictions raised by previous investigations into ciliary epithelial transport. In order to accomplish this, the isolated I-CB of the albino rabbit will be used to investigate the ion transport properties of the ciliary epithelium. Because of the unique configuration of its two different epithelial

layers juxtaposed by their apical sides, the ciliary body is a particularly challenging membrane to study.

The techniques to be used are essentially similar to those used in other epithelia (frog skin, toad bladder, cornea, etc.) to study transport processes. As in the experiments of Ussing and Zerahn, the tissue is used to separate two chambers (containing oxygenated Tyrode Ringer) and is arranged so that the stromal side of the I-CB faces one chamber and the epithelial side the other. (Figure IV)

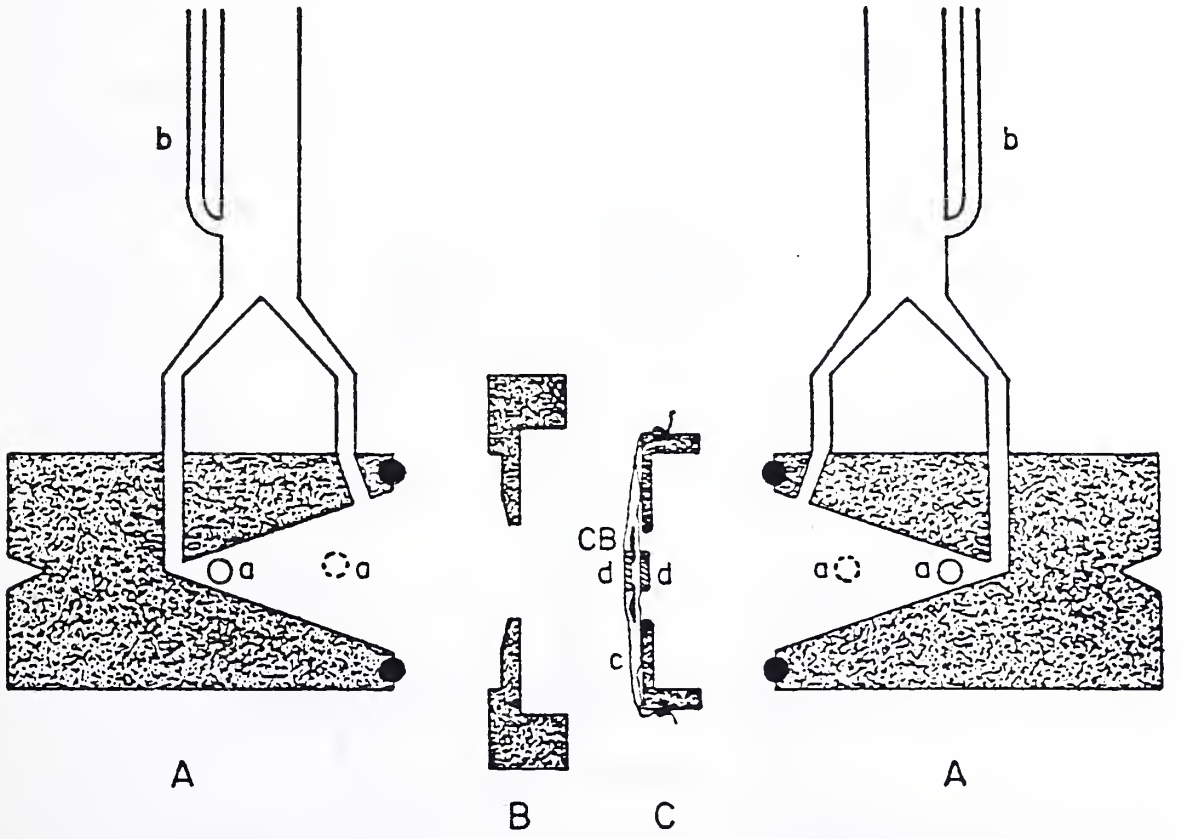

Figure IV. Diagram of the chamber built for I-CB trans-epithelial electrical measurements. Lucite chambers (A) contain ports (a) for PD and SCC bridges and a glass bubbler (b). Outer Lucite mounting block (B) with central 12mm diameter openings. Inner Lucite mounting block (C) contains mounted iris-ciliary body (CB) fixated in place by nylon mesh (c). Lucite discs (d) occlude the pupil. Rubber O-rings are shown as darkened circles. See text of manuscript for sequential description of the mounting of the membrane in this chamber. (From KRUPIN, 1984)

Such a system makes possible the transepithelial measurement of electrical PD, SCC, and unidirectional ion fluxes. It also allows the examination of the direct effects of pharmacological agents and metabolites on these aforementioned parameters.

B. Proposed Model of Ciliary Epithelial Transport

Based on preliminary findings in the laboratory of Oscar A. Candia, M.D. that have been recently published in Krupin, et al.,[26] a hypothetical model of the transport system of the two cell layers of the I-CB has been proposed (FIGURE V). This model is also consistent with the findings of other investigators.[34,37]

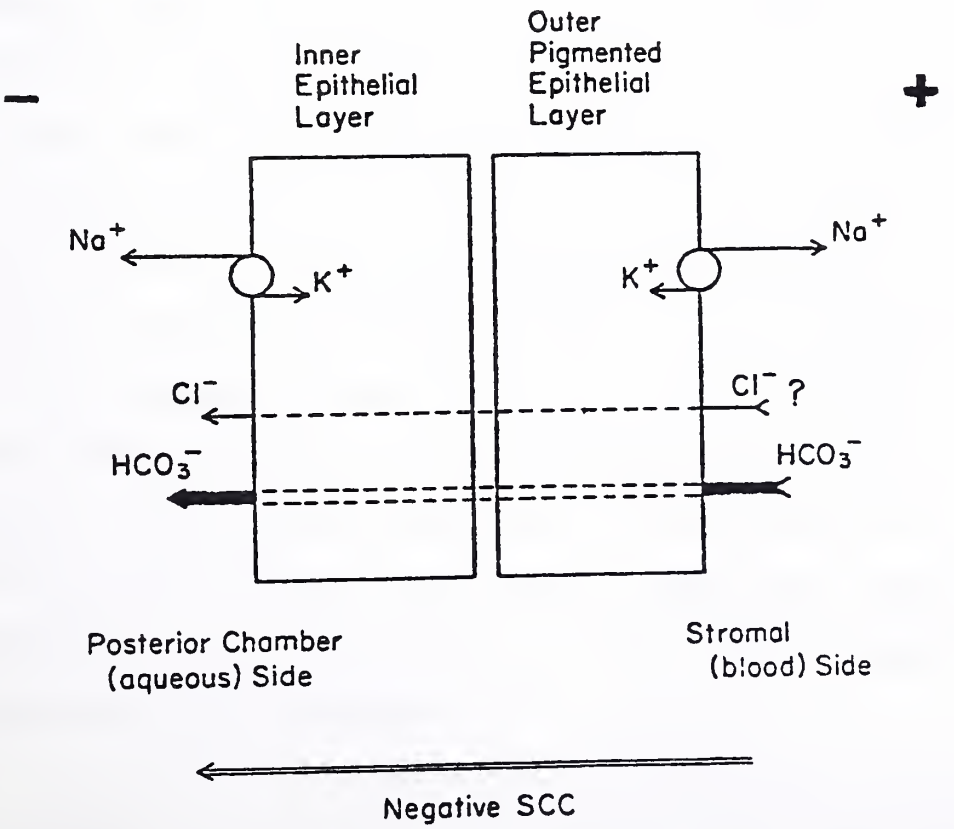

FIGURE V. Recently proposed model of rabbit ciliary epithelial transport system. (From KRUPIN, 1984)

Note the unique configuration of the ciliary epithelium consisting of two distinct epithelial cell layers electrically connected by gap junctions. Their apical sides are directed toward each other, and their basolateral membranes face the blood (pigmented) and aqueous (non-pigmented) sides. Since anatomic coupling between the two cell layers of the I-CB has been identified,[5] electrical coupling may also exist between these two layers.

C. Na^+-K^+ Pumps and the Effect of Ouabain

The dependence of active ionic transport on oxidative metabolism has been demonstrated by the fact that anoxia reduces the PD and SCC of the I-CB.[26] Ouabain, a known potent inhibitor of Na^+-K^+ ATPase in several epithelial membrane systems, inhibits I-CB oxygen consumption. A similar decline in respiratory rate results when I-CB's are bathed in Na^+ -free or K^+ -free solutions, and ouabain has no additional inhibitory effect in either of these solutions.[26] The accepted location of the ouabain binding site in the ciliary epithelium is the basolateral membrane of the non-pigmented cell layer.[40] However, more recent experiments using strontium as a capture ion for Na^+-K^+ ATPase localization have shown enzyme reaction products in the basal surface of both the non-pigmented and pigmented ciliary epithelia.[41]

The results of experiments investigating the effect of ouabain on the SCC of the I-CB are also consistent with a double location of the Na^+-K^+ pump sites.

Addition of 5×10^{-5}M ouabain to the aqueous side chamber increases the SCC initially, with subsequent decline to zero. Ouabain (5×10^{-5}M) added to the blood side results in an SCC inhibition. The different time-course of the inhibitory effect of ouabain when the drug is added to either side, including the initial stimulation of the SCC when ouabain is added to the aqueous side, can be understood by examination of Figure V with the following in mind. Ouabain added to the blood side inhibits the Na^+-K^+ pump there, resulting in the immediate reduction of SCC and inhibition of all transport processes. Ouabain added to the aqueous side first inhibits the aqueous Na^+-K^+ pump, causing an immediate rise in the SCC. Then, by either diffusion or transport[42] through both layers, ouabain inhibits the blood-side Na^+-K^+ pump and all transport processes, resulting in a declining phase of the SCC.[26] The rates of Na^+ transport by the two basolateral Na^+-K^+ pumps need not be identical to explain the side-dependent and biphasic effects of ouabain. Note that ouabain-sensitive sites have also been suggested to be present on both the aqueous and blood sides of I-CB preparations of the toad.[37]

D. HCO_3^- Transport

Bilateral ionic substitution experiments on the SCC have revealed that the SCC is totally dependent on Na^+, K^+, and HCO_3^-. An interaction or coupling among these three ions is suggested by the fact that absence of just one of these three ions produces a complete inhibition of the PD and SCC. While coupling is a well-known phenomenon, whether and in what

way HCO_3^- interacts with Na^+ and/or K^+ is unclear at the present time. Note that bilateral HCO_3^- removal experiments (with maintenance of constant pH) suggest a direct involvement of HCO_3^- in establishing the aqueous side negative PD.[26] Transport of HCO_3^- from blood-to-aqueous direction has also been suggested by experiments that showed the HCO_3^- concentration in rabbit posterior chamber aqueous humor to be higher than that in plasma.[6] The measured PD orientation of the aqueous side negative is consistent with these concepts.

E. Cl^- Transport

Active Cl^- transport has been reported to be present in the I-CB of the cat,[36] toad,[38] and rabbit.[34] However, the effects of chloride removal and isosmotic bilateral substitution with SO_4^{-2} have only a minimal inhibitory effect on SCC.[26]

F. Orientation of PD

When identical solutions are in both chamber sides, the PD of the aqueous side of the rabbit I-CB is consistently negative with respect to the blood side. Whereas the measured transport PD is low, the calculated electrical resistance for the unfolded tissue is high.[26] The PD orientation and the SCC direction suggest that there exists either (1) a net translocation of negative charges towards the aqueous humor from the blood side, (2) a net movement of positive charges in the opposite direction, or (3) both conditions co-existing. The most attractive possibility is the first, since *in situ* fluid flow secondary to electrolyte transport is in the blood-to-

aqueous direction.

G. Summary of Recent Findings

Thus, the proposed model shown in Figure V is based on the current findings discussed above. In Figure V, the two locations of the Na^+-K^+ pumps, the net HCO_3^- transport and possible net Cl^- transport are indicated. Only the direction of HCO_3^- and Cl^- transport is indicated, since the location of these pumps is uncertain. The measured SCC from blood-to-aqueous side will equal the sum of the HCO_3^- flux, plus Cl^- flux, plus the net Na^+(minus K^+) fluxes towards the blood side, minus the net Na^+(minus K^+) fluxes towards the aqueous side. Electrical coupling probably exists between the apical membranes and may also be between lateral membranes within each epithelial layer.[26]

The orientation of the PD and SCC and the effects of ouabain and ionic replacement allow one to hypothesize a model to describe the mechanisms of active transport in the I-CB. However, additional studies are needed to further elucidate the transport system of this complicated membrane.

H. Experimental Design of Thesis

Unidirectional Na^+ and Cl^- fluxes were measured across the isolated rabbit I-CB. These results were compared with the electrical resistance by means of the partial conductance equation. This provides an estimate of the fraction of the unidirectional fluxes that crosses the tissue paracellularly. Unidirectional fluxes were measured by adding ^{22}Na and ^{36}Cl to either the stromal or aqueous side of the chamber.

Samples were analyzed in a radioactive counter with results corrected for background and quenching and the fluxes determined. The effect of ouabain on the steady state ionic flux by its addition to either side of the chamber was investigated.

The use of inhibitors of metabolic and transport processes can offer insight into the nature of the secretory process. The following agents were investigated for their effects on transmembrane short-circuit current:

(a) Ouabain, a potent inhibitor of Na^+-K^+ ATPase;

(b) Amphotericin B (Fungizone), a polyene antibiotic which stimulates sodium transport and increases membrane permeability;

(c) Calcium ionophore A23187, a calcium channel antibiotic which enhances calcium influx;

(d) Epinephrine;

(e) Theophylline and iso-butyl methylxanthine (IBMX), inhibitors of phosphodiesterase;

(f) Furosemide, an inhibitor of active chloride transport;

(g) Trifluormethazolamide, a carbonic anhydrase inhibitor; and

(h) DIDS (di-iso-thiocyanostilbene disulfonic acid), an inhibitor of the anion exchange system (including bicarbonate).

Many of the above agents have previously been found to raise or lower intraocular pressure <u>in vivo</u>. However, it has not been certain whether they are acting directly on aqueous humor secretion, aqueous humor outflow, or ocular blood flow. The Ussing-type chamber is an excellent means to examine such questions.

The experimental work for this thesis was accomplished from June through August, 1982 in the laboratory of Oscar A. Candia, M.D., Professor of Ophthalmology and Associate Professor of Physiology and Biophysics at Mount Sinai School of Medicine in New York, New York. All the research was done by me at Mount Sinai, and all expenses for research equipment, materials, and animals were supplied by Dr. Candia. My investigative portion of this thesis has been published in the refereed journal Current Eye Research, 2:815-827, 1983. Following "Literature Cited," Section V of this thesis, is a detailed analysis of my original research presented as a manuscript reprint from that journal. It includes sections entitled Introduction, Materials and Methods, Results, Discussion and References. It represents my effort to better elucidate the mechanisms fundamental to aqueous humor secretion.

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Na^+ and Cl^- fluxes, and effects of pharmacological agents on the short-circuit current of the isolated rabbit iris-ciliary body

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ABSTRACT

Unidirectional Na^+ and Cl^- fluxes were measured across the isolated rabbit iris-ciliary body under short-circuited conditions. Na^+ fluxes were in the range of 9-13 $\mu eq/hr \cdot cm^2$, and Cl^- fluxes varied between 7-12 $\mu eq/hr \cdot cm^2$. A statistically significant net Na^+ or Cl^- flux could not be found. Ouabain, $5X10^{-5}$ M, did not change the Na^+ or Cl^- flux in either direction despite a marked effect on the short-circuit current (SCC). There was a disagreement between the electrical conductance calculated from unidirectional fluxes and electrical measurements, suggesting the presence of electrically silent exchange mechanisms. Theophylline and isobutyl methylxanthine stimulated the SCC, whereas epinephrine, trifluormethazolamide and diisothiocyanostilbene disulfonic acid inhibited the SCC. Furosemide had a minor inhibitory effect, and the Ca^{2+} ionophore A23187 was without effect. Amphotericin B produced a substantial stimulation of the SCC from the aqueous side but an inhibition of the SCC from the blood side. This dual effect is consistent with the presence of Na^+-K^+ pumps in the basolateral membranes of both the pigmented and non-pigmented cell layers of the ciliary body.

INTRODUCTION

Aqueous humor is produced by a net fluid movement across the epithelial layers of the ciliary processes. Part of this fluid movement occurs as a result of secretion, a mechanism involving fluid movement secondary to active ionic transport. To characterize ion pumps contributing to aqueous humor secretion, the in vitro isolated iris-ciliary body (I-CB) has been used in the past by several investigators (1-14). Although early reports by Cole (1,2,4) indicated a transepithelial potential difference (PD) positive on the aqueous side of the rabbit I-CB, more recent work with the I-CB from the same animal by Kishida et al. (11-13) and Krupin et al. (14) show the aqueous side consistently about 1 mV negative. Krupin et al. (14) had postulated that the PD and associated short-circuit current (SCC) are the result of the algebraic contribution of several possible ion pumps located in the various cell membranes of this complex epithelium.

In this study, we determined the effects of ouabain on unidirectional Na^+ and Cl^- fluxes and investigated the effects of several other pharmacological agents on the SCC in order to isolate the ionic components of the SCC.

MATERIALS AND METHODS

Adult albino rabbits weighing 3-4 kg were sacrificed using air injection into the marginal ear vein or heart. Excised eyes were immediately placed in a modified Tyrode's solution pre-bubbled with 95% O_2:5% CO_2 to assure a pH of 7.5. This solution contained in mM: NaCl 103, KCl 4, $MgCl_2$ 1.2, $CaCl_2$ 1.8, $NaHCO_3$ 30, NaH_2PO_4 0.8, glucose 5.6. The total osmolarity was about 290 mOsm. The ionic composition of this solution resembles that of rabbit aqueous humor. For experiments in HCO_3^--free medium, 39 mM Trizma HCl and 11 mM Trizma base (Sigma Chemical Co., St. Louis, MO) were substituted for $NaHCO_3$, the solution was bubbled with air, and the pH was 7.5.

I-CB dissection and securing of the tissue between two Lucite mounting block halves were performed using the technique previously described by Krupin et al. (14). The globe was bisected 5 mm posterior to the limbus. The anterior half was placed in Tyrode's solution with the corneal surface down. The choroid-retina was separated from the sclera to the ora serrata, the lens and its capsule were removed by cutting the zonules, and the ciliary body was separated from the sclera with a lamellar dissecting blade. The I-CB was transferred on a flat spatula and placed on a

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platform which consisted of a two-piece circular Lucite block. The inner block had a central opening 12 mm in diameter with a recessed rubber O-ring. Nylon stocking was stretched tightly over the block's surface and held in place with an outer rubber O-ring. The dissected I-CB was centered, using the choroid-retina for manipulation, over the central block opening. A 7 mm diameter Lucite disc was attached to the posterior surface of the iris using cyanoacrylate adhesive (Eastman 910) to occlude the pupil. A second piece of nylon stocking was gently placed over the tissue. The outer mounting block with a matching central 12 mm diameter opening was placed over the inner block. The meshed inner-outer block was turned over and a second Lucite 7 mm disc glued over the area of the first disc, on the nylon facing the anterior iris surface. Therefore, the tissue preparation consisted from the anterior iris surface of the following: Lucite disc, adhesive, nylon, I-CB, adhesive, Lucite disc, and nylon. The exposed tissue surface area was an annulus of 0.75 cm^2. The completed tissue-mounted block was placed between two halves of an Ussing-Zerahn type chamber. The membrane PD was monitored through agar-0.45% NaCl-filled polyethylene bridges connected to saturated KCl-calomel half-cells. The tips of the PD bridges were placed within 1 mm of the I-CB preparation. External current was passed through the I-CB via agar-0.45% NaCl-filled polyethylene bridges. Both the PD and SCC bridges had been pre-soaked overnight in the appropriate bathing solution, either HCO_3^--rich or HCO_3^--free Tyrode's. The membrane PD was continuously short-circuited with an automatic voltage clamp apparatus, and the SCC was measured with a Heath EU-20 recorder. Transepithelial electrical measurements were obtained with each half chamber filled with 10 ml Tyrode's maintained at 37°C and bubbled with 95% O_2:5% CO_2. For PD measurements, the blood side solution (on the pigmented cell layer side) is taken as the reference side. The SCC is considered to be positive when positive charges move across the I-CB from aqueous to blood side. Because of this convention, a negative PD will generate a positive SCC.

When electrical parameters were studied in HCO_3^--free solution, the following modifications were made: PD and SCC bridges were pre-soaked overnight in HCO_3^--free Tyrode's; the I-CB (after dissection in regular Tyrode's) was pre-soaked twice in HCO_3^--free Tyrode's for 20 min; and the bathing solutions in the chambers were bubbled with air.

Unidirectional Na^+ and Cl^- fluxes were measured by adding about 5μCi of ^{22}Na (New England Nuclear, Boston, MA) or 5μCi of ^{36}Cl (Amersham Radiochemicals, Arlington Hts., IL) to one chamber compartment and taking periodic samples from the opposite compartment. The surface of the I-CB exposed to the solutions was 0.75 cm^2. Specific activity in the "hot side" remained constant throughout the experiment, and the activity in the "cold side" was always about 0.001 of that in the hot side. Every 15 min, 2 ml samples were taken from the cold side whose total volume was kept constant at 10 ml. Samples of 50 μl were taken from the hot side and diluted to 2 ml with Tyrode's before counting.

Samples for ^{36}Cl activity measurement were mixed with a modified Bray's solution and counted with a Packard Tri-Carb liquid scintillation spectrometer. ^{22}Na samples were counted with a well scintillation detector connected to the Packard spectrometer.

The following pharmacological agents were used in this study: ouabain octahydrate, L-epinephrine bitartrate, 3-isobutyl-1-methylxanthine (IBMX), and theophylline (Sigma Chemical Co., St. Louis, MO); calcium ionophore A23187 (Calbiochem-Behring Corp., La Jolla, CA); furosemide (Hoechst Pharmaceuticals, Inc., Somerville, NJ); 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) (Pierce Chemical Co., Rockford, IL); amphotericin B (E. R. Squibb, Princeton, NJ); and trifluormethazolamide (TFM) (kindly provided by Dr. Thomas Maren).

Concentrated stock solutions of the drugs were prepared and added to the chamber in a 1 ml volume

after previous removal of 1 ml from the chamber; except for furosemide (0.33 ml), epinephrine (100 µl), amphotericin B (25 µl), and A23187 (20 µl in ethanol). It was determined that 20 µl of ethanol has no effect on the I-CB electrical parameters.

RESULTS

Unidirectional Na^+ fluxes, effect of ouabain

Unidirectional Na^+ fluxes were measured across nonpaired short-circuited I-CB in both directions. The effect of $5X10^{-5}$ M ouabain on these unidirectional fluxes when added either to the blood side solution or to the aqueous side solution was examined. The results of four different experimental conditions are shown in Table 1.

Samples were taken every 15 min, and experiments were usually run for 4 hours. Ouabain was added to the bathing solution after at least four stable measurements were obtained. In analyzing the ouabain effect on Na^+ fluxes, four determinations prior to the addition of ouabain were compared with four determinations following the ouabain addition. No change or a slight increase in the unidirectional fluxes was observed after ouabain in all the experimental conditions.

As reported previously (12,14) and shown in Fig. 1, in this study ouabain consistently stimulated the SCC by 5.4±0.6 $\mu A/cm^2$ (n=16) when added to the aqueous side and reduced the SCC by 6.7±0.7 $\mu A/cm^2$ (n=15) when added to the blood side. The stimulation lasted for about 50 min.

Table 1: Effects of $5X10^{-5}$ M ouabain on sodium fluxes (µeq/hr·cm^2) from blood to aqueous side (Bl→Aq) and aqueous to blood side (Aq→Bl) in isolated rabbit ciliary body

Successive 15 min periods	Na flux Bl→Aq (n=5)	Na flux Aq→Bl (n=5)	Successive 15 min periods	Na flux Bl→Aq (n=5)	Na flux Aq→Bl (n=4)
Control	7.1 ± 1.1	7.6 ± 3.1	Control	8.8 ± 1.6	6.8 ± 1.9
"	8.1 ± 1.7	7.0 ± 1.6	"	12.8 ± 1.7	9.1 ± 1.6
"	*10.0 ± 2.0	* 9.4 ± 1.0	"	*12.9 ± 0.9	* 9.0 ± 1.5
"	*10.8 ± 2.2	*11.0 ± 1.1	"	*13.1 ± 0.8	* 9.2 ± 1.3
"	*10.0 ± 1.7	*10.2 ± 1.2	"	*13.2 ± 0.9	* 9.7 ± 1.4
"	*10.4 ± 1.7	*10.4 ± 1.1	"	*12.9 ± 0.8	* 9.0 ± 1.6
Ouabain	†10.7 ± 1.7	† 9.9 ± 1.1	Ouabain	†13.0 ± 0.9	† 9.5 ± 1.3
Aq. side	†11.7 ± 2.0	†10.0 ± 1.4	Bl. side	†12.5 ± 1.0	†10.6 ± 2.0
"	†12.0 ± 2.3	†11.2 ± 1.3	"	†14.7 ± 1.6	†11.1 ± 1.8
"	†13.3 ± 2.4	†11.0 ± 1.3	"	†15.7 ± 1.7	†11.6 ± 1.1
"	12.8 ± 2.1	10.8 ± 1.2	"	14.5 ± 1.9	11.6 ± 1.5
"	13.1 ± 2.4	11.4 ± 2.0	"	14.3 ± 1.4	11.7 ± 1.3
"	13.6 ± 2.3	11.6 ± 1.3	"	13.8 ± 1.6	10.8 ± 1.2
"	12.6 ± 2.2	12.2 ± 1.4	"	13.6 ± 1.5	11.4 ± 1.4
			"	13.5 ± 2.0	10.9 ± 1.9
			"	13.5 ± 2.2	11.6 ± 1.3

Each value in the table is the Mean ±SE of corresponding periods from (n) experiments.

*Mean ±SE of 4 control periods:	10.3 ± 0.2	10.3 ± 0.3	°13.0 ± 0.1	9.2 ± 0.2
†Mean ±SE of 4 ouabain periods:	11.9 ± 0.5	10.5 ± 0.3	°14.0 ± 0.7	10.7 ± 0.4×

×Ouabain periods significantly larger than corresponding control periods; $p > 0.05$.
°Bl→Aq larger than corresponding Aq→Bl fluxes; $p < 0.05$.

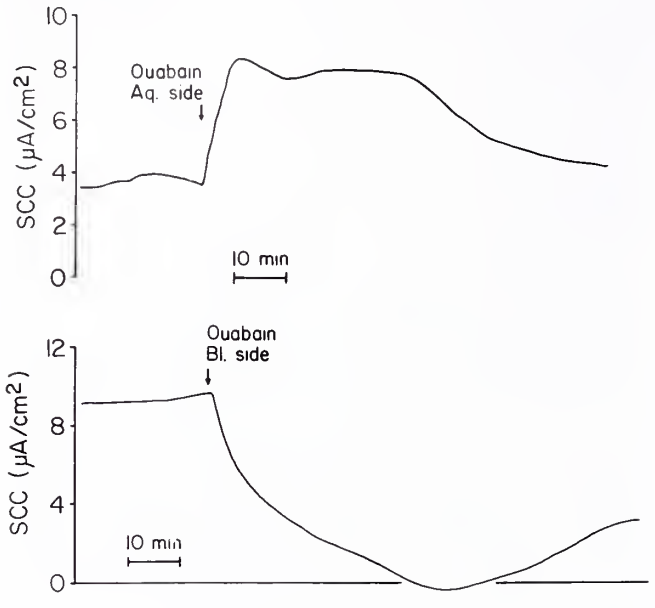

Figure 1: Effect of ouabain ($5X10^{-5}$ M) on the SCC of the isolated rabbit I-CB. Upper panel: ouabain addition to the aqueous (Aq.) side. Lower panel: ouabain addition to the blood (Bl.) side.

These changes were not reflected in the unidirectional flux values.

In comparing the first two sets of experiments (ouabain on aqueous side, Table 1), we could not detect a significant net Na^+ flux either before or after ouabain. In the other two sets (when ouabain was added to the blood side), a small net flux in the blood to aqueous direction was seen before ouabain. This net difference was reduced by ouabain. It should be noted that whereas the SCC ranges from 4-8 $\mu A/cm^2$ and the change in SCC after ouabain is 5-7 $\mu A/cm^2$, the unidirectional Na^+ fluxes carry a current equivalent to about 300 $\mu A/cm^2$. Thus, only a small fraction of the unidirectional Na^+ flux seems to move via the Na^+-K^+ pump, and little change should be expected from an ouabain inhibition.

Unidirectional Cl^- fluxes, effect of ouabain

The effect of ouabain on unidirectional Cl^- fluxes across the short-circuited I-CB was also examined. The protocol and analysis of the data were similar to that described for Na^+ fluxes and the results are shown in Table 2.

Since unidirectional Cl^- fluxes were relatively high and only a small net flux, if any, was expected (12,14), our main interest was to determine a change in the unidirectional fluxes by ouabain. Thus, only six experiments were performed. Consistent with the previously described small net Cl^- transport from blood to aqueous (13), the average blood to aqueous Cl^- flux (10.9±1.0) was slightly larger than the oppositely directed flux (9.2±1.0). Ouabain had no significant effects on the unidirectional Cl^- fluxes in five experiments but produced an increase on the flux in one case. The value of the unidirectional Cl^- fluxes was of the same order of magnitude as that of the Na^+ fluxes.

Since only a small fraction of the unidirectional Na^+ and Cl^- fluxes represents a net flux (pump component), it can be assumed that most of the Na^+ and Cl^- moves across the I-CB via the diffusional pathway. If this is the case, electrical conductance measured as $\Delta I/\Delta V$ should agree with that calculated from the partial conductance equation using the unidirectional flux measurements. Average electrical conductance from all experiments in which unidirectional Na^+ or Cl^- fluxes were measured was 8.33±0.91 mS/cm^2. However, conductance calculated from unidirectional Na^+ and Cl^- fluxes was 22.1 mS/cm^2, a value 2.7 times larger than the conductance calculated from the electrical measurement. Possible reasons for this discrepancy are analyzed in the Discussion.

Effect of ouabain on the I-CB in HCO_3^--free Tris Tyrode's

Both Kishida et al. (12) and Krupin et al. (14) have reported a PD negative on the aqueous side only when HCO_3^- is present in the bathing solutions. Krupin et al. interpreted the value and polarity of the PD as the result of a combination of several pump mechanisms present in the two cell layers. Thus, it follows that the absence of a PD does not necessarily mean a non-viable preparation. Therefore, even in the absence of HCO_3^-, without any appreciable PD, ouabain should modify the SCC as observed in

Table 2: Effects of $5X10^{-5}$ M ouabain on chloride fluxes (μeq/hr•cm^2) from blood to aqueous side (Bl→Aq) and aqueous to blood side (Aq→Bl) in isolated rabbit ciliary body

Successive 15 min periods	Cl flux (Bl→Aq)			Cl flux (Aq→Bl)		
Control	8.4	12.0	8.9	Control 8.7	5.4	5.0
"	9.0	12.0	12.1	10.6	6.9	8.6
"	*8.8	*11.5	*11.8	*10.4	*7.8	* 9.3
"	*9.3	*11.6	*12.7	* 9.8	*6.9	*10.0
"	*9.1	*12.5	*12.8	* 9.9	*7.0	* 9.6
"	*8.4	*10.9	*11.8	*11.8	*7.4	* 9.9
Ouabain	†9.4	†11.8	Ouabain †12.4	Ouabain †10.3	Ouabain †6.8	†11.1
Aq. side	†8.0	†11.5	Bl. side †11.7	Aq. side †10.9	Bl. side †7.6	†11.4
"	†9.9	†11.2	†13.5	†10.5	†8.4	†11.4
"	†7.4	†11.9	†13.1	†11.7	†8.5	†12.6
"	9.5	10.2	10.7	11.2	8.5	14.3
"	8.2	12.6	13.5	11.3	8.7	11.6
"	9.2	11.9	11.0	9.9	8.3	12.7
"	8.2	10.2	11.3	11.1	8.6	12.1
"	8.7	11.0	11.2	10.6	8.8	11.3
*Mean ±SE of 4 control periods:	8.9±0.2	11.6±0.3	12.3±0.3	10.5±0.5	7.3±0.2	9.7±0.2
†Mean ±SE of 4 ouabain periods:	8.7±0.6	11.6±0.2	12.7±0.4	10.9±0.3	7.8±0.4	11.6±0.3×

×Significantly larger than corresponding control periods; $p < 0.05$.

HCO_3^--rich bathing solution. As shown in Table 3, ouabain on the aqueous side increased the SCC from -0.5 μA/cm^2 to 6.3 μA/cm^2, a statistically significant change of 6.8 μA/cm^2. Ouabain on the blood side induced a statistically significant negative current (positive charges from blood to aqueous) change of -2.3 μA/cm^2. Two experiments are illustrated in Fig. 2. As seen in HCO_3^--rich solution, ouabain exerts the same dual effect in HCO_3^--free solution, and the final SCC after the drug has been added to both sides is positive. It should be noted that there was no difference between the stimulation produced by ouabain on the aqueous side in bicarbonate-rich or bicarbonate-free solution; whereas the inhibitory effect when ouabain was added to the blood side in bicarbonate-free solution was significantly smaller than in bicarbonate-rich solution (2.3±0.5 vs. 6.7±0.7; $p < 0.01$).

Effects of pharmacological agents on the SCC across the I-CB

Cellular calcium concentration has been implicated in the regulation of ionic transport in several systems (15). The calcium ionophore A23187 has been shown to increase cellular calcium concentration and mimic the effect of epinephrine, cAMP, and phosphodiesterase inhibitors in stimulating active Cl^- transport in the cornea (16) and other epithelia (17). Furthermore, Podos (18) has shown that topical application of the ionophore increased intraocular pressure (IOP). In our experiments, A23187 (10^{-5} M) in either or both bathing solutions failed to produce any modification in the PD or SCC of the I-CB. Similarly, epinephrine in the aqueous side solution in concentrations as high as 10^{-5} M did

Table 3: Sequential changes in SCC ($\mu A/cm^2$) by $5X10^{-5}$ M ouabain in isolated rabbit ciliary body in bicarbonate-free Tyrode's

	ΔSCC after addition to	
	*Aq. side	Bl. side
	13.3	-9.3
	4.3	-1.3
	6.7	-0.8
	4.0	-4.0
	5.9	-2.7
Mean ±SE	6.8 ± 1.7	-3.6 ± 1.5

	†Bl. side	Aq. side
	-2.5	5.6
	-3.2	4.5
	-4.0	4.8
	-3.5	5.3
	0.0	2.9
	-0.4	4.7
	-1.6	2.4
	-3.5	5.9
Mean ±SE	-2.3 ± 0.5	4.5 ± 0.4

*Ouabain was added to aqueous side (Aq.) first and maximum change recorded. Then ouabain was added to blood side (Bl.) and a decrease in SCC recorded.
†Ouabain was added to blood side first and maximum change recorded. Then ouabain was added to aqueous side and an increase in SCC recorded.
All Mean values are different from zero. $p < 0.05$ for -3.6. $p < 0.01$ for all other Means.

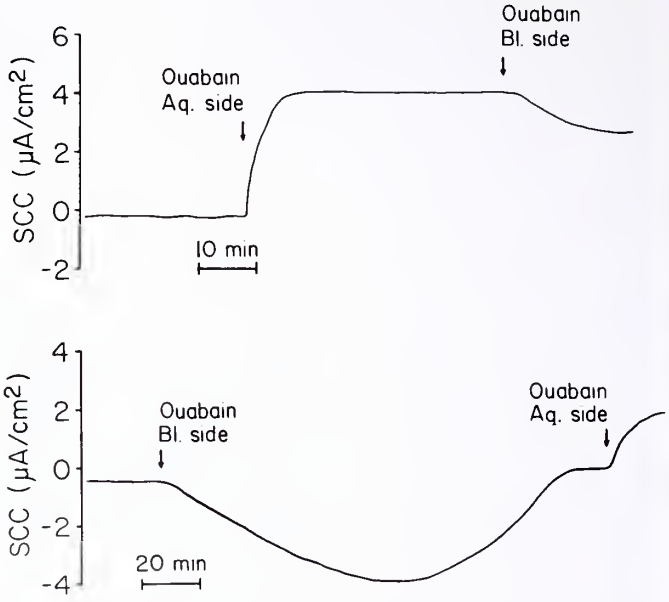

Figure 2: Sequential effect of ouabain ($5X10^{-5}$ M) on the SCC of the isolated rabbit I-CB bathed in HCO_3^--free Tyrode's solution. Upper panel: ouabain added to the aqueous (Aq.) side followed by ouabain to the blood (Bl.) side. Lower panel: ouabain added to the blood side followed by ouabain to the aqueous side.

not affect the PD or SCC. However, it consistently inhibited the SCC by 1.6 $\mu A/cm^2$ when added to the blood side solution.

Theophylline (1 mM) on the aqueous side stimulated the SCC by 1.3 $\mu A/cm^2$, whereas there was no significant effect when it was added to the blood side. IBMX (2 mM) stimulated the SCC only slightly when added to the aqueous side solution but significantly from the blood side (Table 4).

Kishida et al. had reported an inhibitory effect of furosemide on the SCC of the rabbit I-CB (12), and found a net Cl^- transport from blood to aqueous that presumably represents the SCC fraction inhibited by furosemide (13). In this study, furosemide at high concentrations (1 mM) had only a minor inhibitory effect when added to the blood side and no effect when added to the aqueous side (Table 4). Similarly, in the few experiments in which furosemide was tested in HCO_3^--free solution, the decrease in SCC was only 1 $\mu A/cm^2$.

The carbonic anhydrase inhibitor, TFM, in a 1mM concentration, produced a decrease in the SCC of 2 $\mu A/cm^2$ after addition to the solutions on both sides (Table 4).

Disulfonic stilbenes are known to inhibit anion exchange systems present in many cells (19-21). Anion exchange systems may be present in one or both cell layers of the I-CB and may contribute to the SCC. Thus, we tested the effect of the disulfonic stilbene, DIDS, at 10^{-4} M concentration. In the blood side solution, DIDS had no effect on the electrical parameters in either HCO_3^--rich or HCO_3^--free solution. However, there was a clear inhibition of the SCC

Table 4: Average changes in SCC ($\mu A/cm^2$) by various agents in isolated rabbit ciliary body bathed in Tyrode's

Theophylline 1 mM (Aq.) n=14	Theophylline 1 mM (Bl.) n=11	IBMX 2 mM (Aq.) n=3	IBMX 2 mM (Bl.) n=3
1.3 ± 0.6†	0.2 ± 0.2	0.5 ± 0.3	2.2 ± 1.0†

Epinephrine 10^{-5} M (Bl.) n=3	Furosemide 1 mM (Bl.) n=5	TFM 1 mM (Aq.) n=5	TFM 1 mM (Bl.) n=5	DIDS 10^{-4}M (Aq.) n=8
-1.6 ± 0.5†	-0.5 ± 0.2†	-1.5 ± 0.6†	-0.5 ± 0.3	-2.5 ± 0.6†

"Aq." indicates addition to aqueous side solution.
"Bl." indicates addition to blood side solution.
All values are Mean ±SE.
†Changes significantly different from zero; $p < 0.05$.

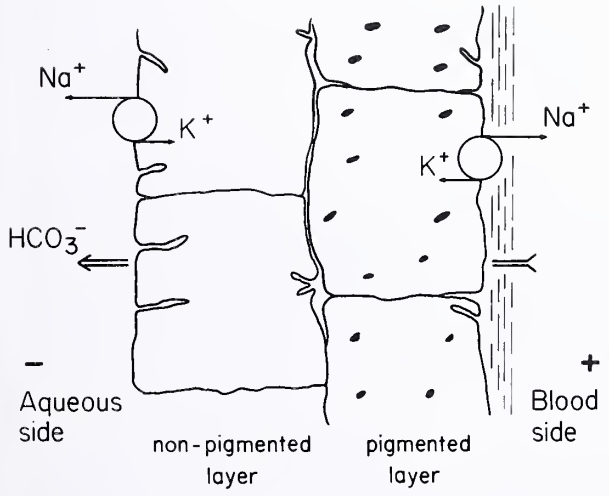

Figure 3: Schematic diagram of ionic transport across the isolated rabbit ciliary epithelium.

by DIDS in the aqueous side solution. In HCO_3^--rich solution, the average SCC decrease was 2.5 $\mu A/cm^2$ (Table 4); whereas, in HCO_3^--free solution, the average SCC decrease was 0.67 $\mu A/cm^2$.

Effect of amphotericin B on the electrical parameters across the I-CB

It has been postulated previously that the stimulation and inhibition of the SCC by ouabain were due to its effect on the Na^+-K^+ pump of the non-pigmented layer and pigmented layer, respectively (14) (Fig. 3). Amphotericin B is known to stimulate active ionic transport in many epithelial systems when added to the apical side solution. It acts by largely increasing the apical membrane permeability to ions and by making the operation of the pump on the basolateral membrane more efficient (22,24). In the I-CB preparation, the Na^+-K^+ pump on the basolateral membrane of the non-pigmented layer is in direct contact with the bathing solution. If amphotericin B creates channels in parallel with the pump in one basolateral membrane, it should indirectly stimulate active transport by the pump located in the opposite basolateral membrane. Thus, the effect of amphotericin B (10^{-5} M) was tested by adding it to either the aqueous or the blood side solution. As shown in Fig. 4, amphotericin B produced a large increase in the SCC when added to the aqueous solution. It should be noted that, unlike ouabain, amphotericin B caused an SCC stimulation that remained stable for several hours. This may be interpreted as an increase in the Na^+ pumping rate of the pigmented

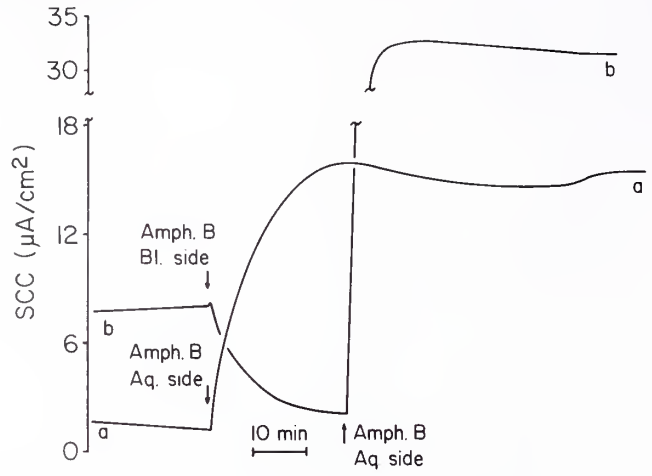

Figure 4: Effect of 10^{-5} M amphotericin B (Amph. B) on the SCC of the isolated rabbit I-CB. (a) Amph. B added to the aqueous (Aq.) side. (b) Amph. B added to the blood (Bl.) side followed by Amph. B to the aqueous side.

side Na^+-K^+ pump resulting from the increased permeability of the non-pigmented basolateral membrane. Conversely, amphotericin B added to the blood side reduced the SCC, possibly by facilitating the transfer of Na^+ into the aqueous side solution. The two effects could be seen in sequence when amphotericin B was added to one side solution first and then to the other solution after the first effect had been elicited. As shown in Table 5, the amphotericin B effect on the blood side was larger when the SCC had been stimulated previously by amphotericin B on the aqueous side. Although the stimulation of the SCC was accompanied by a decrease in electrical resistance, there was no significant change in resistance when amphotericin B was added to the blood side solution.

To confirm that the amphotericin B effects were related to Na^+-K^+ pump stimulation independent of a possible HCO_3^- transport, the polyene antibiotic was also tested in HCO_3^--free solution. The effects were similar to those in HCO_3^--rich solution, i.e. a large stimulation when added to the aqueous side and a moderate inhibition when added to the blood side. These results are summarized in Table 5.

As can be seen from the ouabain and amphotericin B experiments, the effects of these drugs on the SCC are qualitatively similar, although their mechanisms of action differ. We also examined the sequential effects of amphotericin B and ouabain. After amphotericin B addition to the aqueous side, ouabain to the aqueous side produced a further, although smaller than usual, stimulation. Also, in experiments where the initial amphotericin B effects were largest, the additional ouabain stimulations were smallest. Ouabain added to the blood side after amphotericin B to the aqueous side produced an SCC inhibition that overcame most, if not all, of the previous amphotericin B stimulation. Since the effect of amphotericin B on the blood side was moderate, subsequent addition of ouabain to the blood or aqueous side elicited a response comparable to that when ouabain was acting alone. In several experiments, the two drugs were added to both sides and in most experiments the four possible individual responses were elicited. The results of some of the combinations of amphotericin B and ouabain are illustrated in Fig. 5.

To see whether the amphotericin B-stimulated SCC included a larger than usual DIDS-inhibitable fraction, the disulfonic stilbene was added to the aqueous side after the SCC was stimulated by amphotericin B on the aqueous side. DIDS inhibition of the SCC was the same as in tissues not treated with amphotericin B. Also, amphotericin B on the aqueous side produced its usual large stimulation after the SCC had been inhibited by DIDS.

It may be possible that Cl^- transport is restricted under normal conditions but facilitated if the membrane permeability has been increased by amphotericin B. Nevertheless, following amphotericin B addition, the effect of furosemide was minimal, as in control conditions.

DISCUSSION

Unidirectional Na^+ fluxes were large, ranging from 9-13 $\mu eq/hr \cdot cm^2$. In one group of experiments

Table 5: Sequential changes in SCC ($\mu A/cm^2$) by 10^{-5} M amphotericin B in isolated rabbit ciliary body in bicarbonate-rich Tyrode's and bicarbonate-free Tyrode's

	ΔSCC in bicarbonate-rich Tyrode's after addition to			
	*Aq. side	Bl. side	†Bl. side	Aq. side
	5.3	- 1.1	-2.4	4.0
	24.0	- 9.3	-3.2	9.9
	11.7	-10.7	-2.0	8.5
	35.6	-17.3	-0.1	8.8
	4.5	- 0.5	-5.9	30.3
	20.4	- 9.7	-7.3	10.5
	15.2			
	11.7			
	25.0			
	13.1			
	12.0			
Mean ±SE	16.2 ± 2.8$^{\Delta}$	-8.1 ± 2.6$^{\Delta}$	-3.5 ± 1.1$^{\Delta}$	12.0 ± 3.8$^{\Delta}$

	ΔSCC in bicarbonate-free Tyrode's after addition to			
	*Aq. side	Bl. side	†Bl. side	Aq. side
	30.8	-4.0	-1.5	12.8
	7.6	0.0	-2.5	10.3
	6.7	-0.7	-2.5	18.3
	17.1	0.0	-5.1	29.3
Mean ±SE	15.5 ± 5.6‡	-1.2 ± 1.0	-2.9 ± 0.8$^{\Delta}$	17.7 ± 4.2$^{\Delta}$

*Amphotericin B was added to aqueous side (Aq.) first and maximum change recorded. Then amphotericin B was added to blood side (Bl.) and a decrease in SCC recorded.
†Amphotericin B was added to blood side first and a maximum change recorded. Then amphotericin B was added to aqueous side and an increase in SCC recorded.
Means significantly different from zero; $^{\Delta}p < 0.02$; $^{\ddagger}p < 0.05$

(Table 1), no net flux could be detected in control conditions; whereas in the other group, an apparent net Na^+ flux of 3.8 $\mu eq/hr \cdot cm^2$ from blood to aqueous can be calculated. Clearly, this net flux is unrealistic since it corresponds to a negative SCC of 100 $\mu A/cm^2$. Using a similar preparation, Kishida et al. (13) have recently reported unidirectional Na^+ fluxes of about 9 $\mu eq/hr \cdot cm^2$ and failed to detect a statistically significant net Na^+ flux. Under nonshort-circuit conditions, Pederson and Green (7) found no net flux of either Na^+ or Cl^- with unidirectional fluxes of a similar magnitude as determined in this study.

Because the unidirectional Na^+ fluxes are high (about 300 $\mu A/cm^2$) and variable, a small difference between these fluxes on the order of the SCC (4-8 $\mu A/cm^2$) may be difficult to detect. Furthermore, Krupin et al. (14) have postulated two opposing Na^+-K^+ pumps in the two layers of the ciliary body, and if these pumps are of the same magnitude, no net flux is expected. Previous ouabain binding studies (25) and the effect of ouabain on the SCC strongly suggest the presence of Na^+-K^+ pumps in the two layers of the ciliary body. In this study, ouabain on either the blood side or the aqueous side failed to produce any significant change in either unidirectional flux,

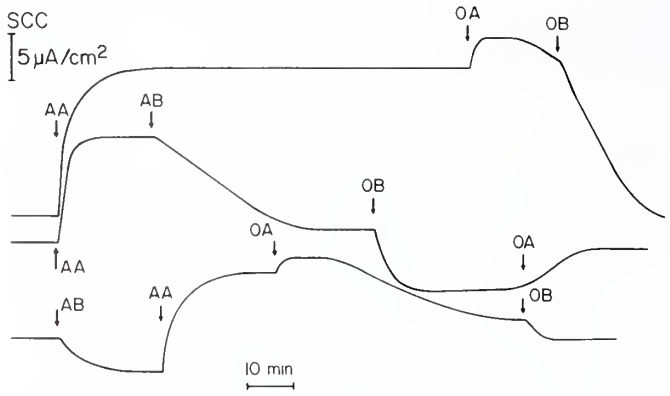

Figure 5: Sequential effects on the SCC of 10^{-5} M amphotericin B added to the aqueous side (AA) or blood side (AB), followed by $5X10^{-5}$ M ouabain added to the aqueous side (OA) or blood side (OB), in three separate experiments.

even though the usual SCC increase (when added to the aqueous side) or SCC decrease (when added to the blood side) was concomitantly observed. It should be noted that the changes in SCC were on the order of 6 $\mu A/cm^2$, corresponding to 0.22 $\mu eq/hr \cdot cm^2$, a change difficult to detect on a 12 $\mu eq/hr \cdot cm^2$ flux.

When ouabain is added to the aqueous side, the SCC is stimulated for about an hour and then begins to decrease. Similarly, the SCC, after having been inhibited by ouabain on the blood side for about an hour, begins to increase. This indicates that ouabain reaches the opposite side of the I-CB after about an hour and begins to inhibit the Na^+-K^+ pump on that side. This ouabain permeation through the I-CB was also suggested by Kishida et al. (12).

Unidirectional Cl^- fluxes were also large (7.3-12.3 $\mu eq/hr \cdot cm^2$), though smaller than those reported by Kishida et al. (about 14.5 $\mu eq/hr \cdot cm^2$) (11,13). Given the large variability of unidirectional fluxes, our limited number of experiments cannot provide statistical significance to the 1.7 $\mu eq/hr \cdot cm^2$ net Cl^- flux in the blood-aqueous direction that we found, but seems to confirm the findings of a net Cl^- transport from blood to aqueous side of 2.25 $\mu eq/hr \cdot cm^2$ by Kishida et al. (13) and 3.5 $\mu eq/hr \cdot cm^2$ by Kishida and Sasabe (11). One must be cautious in interpreting this net Cl^- flux as representing an actual active Cl^- transport across the I-CB for the following reasons: a) removal of Cl^- from the bathing solution has little influence on the SCC (14); and b) in this study, furosemide had only a minor effect on the SCC. Although Kishida et al. have reported a 7 $\mu A/cm^2$ inhibition of the SCC by furosemide (12), this value is only a fraction of their reported 60 $\mu A/cm^2$ net Cl^- flux (13); and, furthermore, furosemide had no effect on unidirectional Cl^- fluxes (11).

Ouabain is known to inhibit active Cl^- transport in epithelia where a Na^+-Cl^- cotransport system is involved. However, in this study, ouabain failed to produce a significant decrease in the Cl^- fluxes in any of six experiments. Disulfonic stilbenes inhibit Cl^- transport in some epithelia (28). In this study, DIDS inhibited the SCC by -2.5 $\mu A/cm^2$, a value also much smaller than the amount expected from the reported net Cl^- fluxes.

Given the large values of the unidirectional fluxes, the variability between preparations, and the net flux expected from the SCC and drug effects, the usefulness of unidirectional fluxes to characterize mechanisms of transport in the I-CB is questionable until more is known about this preparation.

The electrical resistance of the isolated I-CB reported by Krupin et al. was 152 $\Omega \cdot cm^2$ (14). In this study, the mean value from experiments where unidirectional Na^+ and Cl^- fluxes were measured was $120 \pm 13\ \Omega \cdot cm^2$ which correspond to an electrical conductance, G_m, of 8.33 mS/cm^2. The conductance of a membrane can also be evaluated from the sum of the partial ionic conductances:

$$G_m = \sum \left[G_m^{Na} + G_m^{Cl} + G_m^{i} \right]$$

where G_m^{Na} and G_m^{Cl} are the partial conductances of Na^+ and Cl^-, respectively, and G_m^{i} is the conductance of all other ions in the solution. As

previously done (16), the partial conductance of an ion can be calculated from the unidirectional flux of the ion by the equation:

$$G_m^i = \vec{J}_i \, zF/RT$$

where G_m^i is expressed in mS/cm^2, $\vec{J}_i$ is the unidirectional flux of the ion in question in mA/cm^2, and z, F, R and T have their usual meanings.

The combined unidirectional Na^+ and Cl^- fluxes are on the order of 560 $\mu A/cm^2$ which gives a conductance of 22.1 mS/cm^2, about 2.7 times larger than the electrical conductance. Possible contribution of other ions to the ionic conductance will further increase this discrepancy. The obvious conclusion is that about 62% of the Na^+ and Cl^- fluxes move across the membrane by non-diffusional pathways. Active transport, solvent drag, single file diffusion, exchange diffusion and isotope interaction have been proposed as reasons for this type of discrepancy in other systems. Active transport cannot be the reason for the discrepancy in the I-CB, since it seems to represent only a small fraction, not 62%, of the unidirectional fluxes.

Podos (18) has shown that topical application of the calcium ionophore A23187 increases IOP in albino rabbits, presumably by stimulating aqueous humor secretion or by causing an inflammatory response. This study favors the second alternative, since the ionophore had no effect on the electrical parameters of the isolated I-CB.

Epinephrine is known to reduce IOP by influencing outflow channels, but its effect on aqueous humor secretion is controversial. Fluorophotometric studies have shown that epinephrine increases aqueous secretion (26,27). In this study, epinephrine had no effect when added to the aqueous side, suggesting an absence of receptors on the inner non-pigmented layer. A small but consistent reduction in the SCC was observed after addition to the blood side. A direct effect of epinephrine on the I-CB thus requires that the drug enter the circulation. Furthermore, epinephrine's secretion-dependent effect on IOP may be minor compared to that resulting from modification of outflow channels.

The inhibitors of phosphodiesterase, theophylline and IBMX, stimulated the SCC. The effect, however, does not necessarily mean an increase in aqueous humor flow, since it may be due to a reduction in transport of Na^+ (or other positive ions) from blood to aqueous side.

Furosemide had only a minor inhibitory effect on the SCC. Although a Cl^--dependent, furosemide-inhibitable SCC has been described in the isolated toad I-CB (30), and active Cl^- transport has been found in the cat I-CB (5,6), the contribution of this anion to the SCC of the rabbit I-CB remains unclear.

TFM is a carbonic anhydrase inhibitor with an inhibitory constant of 10^{-8} M. Reduction in IOP and aqueous humor secretion by topical application of this drug to rabbit eyes has been reported by Stein et al. (29). Assuming that the reduction in SCC is due to a decrease in HCO_3^- transport from blood to aqueous side, the effect of TFM is consistent with a reduction in aqueous humor secretion. Kishida and Sasabe (11) have indicated a slight decrease in SCC by acetazolamide without an effect on Cl^- fluxes.

Of all drugs that produced an inhibition of the SCC in HCO_3^--rich medium, DIDS had the largest effect, regardless of whether the permeability of the aqueous or blood side membrane had been altered by amphotericin B. At this time we can only speculate that, as reported for red blood cells (19), DIDS blocks either anion exchangers or anion-specific pathways in the cell membrane.

Since the involvement of Cl^- transport as a component of the SCC is dubious, HCO_3^- transport is the most likely anion system inhibited by DIDS. This contention is supported by the minimal effect of DIDS in HCO_3^--free medium.

We used amphotericin B as a tool to partially dissect the properties of the two cell layers of the I-CB. Amphotericin B, by creating channels in the membrane in contact with the solution to which

it is added, will partially shunt pumps present in that membrane and indirectly stimulate pumps in the other cell membranes. Like ouabain, but by a different mechanism, amphotericin B on the aqueous side produced a large SCC increase, presumably by stimulating the Na^+-K^+ pump in the pigmented layer. Unlike ouabain, the stimulatory effect was stable for more than an hour, and in most experiments the stimulation was considerable, up to 35 $\mu A/cm^2$. In general, after stimulation by amphotericin B, ouabain added to the aqueous side had no further effect, suggesting that the Na^+-K^+ pump on the aqueous side was completely shunted. On the blood side, the inhibitory effect of amphotericin B was smaller, and a further inhibition was observed by subsequent addition of ouabain to the same side. This suggests that the increase in permeability was not large enough to completely shunt the Na^+-K^+ pump of the pigmented layer. The effect of amphotericin B in HCO_3^--free medium was similar to that in HCO_3^--rich medium, indicating that the changes in SCC were due to stimulation of pumps not related to HCO_3^- transport.

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VII. THESIS SUMMARY AND CONCLUSIONS

A. Na^+ and Cl^- Fluxes

Unidirectional Na^+ and Cl^- fluxes were measured across the isolated rabbit I-CB in an Ussing-type chamber under short-circuited conditions. A comparison of possible net fluxes with the SCC were made. The effect of ouabain on Na^+ and Cl^- fluxes when added to either the blood side solution or aqueous side solution was studied. Unidirectional ionic fluxes were compared with the electrical resistance by means of the partial conductance equation.

1. Na^+ Fluxes

Unidirectional Na^+ fluxes were large, varying between 9-13 $\mu eq/hr \cdot cm^2$, corresponding to about 300 $\mu A/cm^2$. In one group of experiments, no net Na^+ flux was detected under control conditions; in the other group, an apparent net Na^+ flux of 3.8 $\mu eq/hr \cdot cm^2$ from blood to aqueous side was calculated. However, this net flux is not realistic since it corresponds to a negative SCC of 100 $\mu A/cm^2$. The magnitude of the Na^+ flux and the finding of no net Na^+ flux in the first group of experiments is in agreement with Kishida, et al.[35] under short-circuited conditions, and Pederson and Green[29] (see Literature Cited, Section V) under nonshort-circuited conditions. It should be noted that because the unidirectional Na^+ fluxes were high (about 300 $\mu A/cm^2$) and variable, a small net difference between these fluxes on the order of the SCC (4-8 $\mu A/cm^2$) is difficult to detect.

Ouabain consistently stimulated the SCC by 5.4 $\mu A/cm^2$ when added to the aqueous side and decreased the SCC by 6.7 $\mu A/cm^2$ when added to the blood side. However, ouabain on either the blood side or aqueous side failed to produce any significant change in either unidirectional Na^+ flux. Note that while the SCC ranges from 4-8 $\mu A/cm^2$ and the change in SCC after ouabain is 5-7 $\mu A/cm^2$, the unidirectional Na^+ fluxes carry a current equivalent to about 300 $\mu A/cm^2$. Thus, only a small fraction of the unidirectional Na^+ flux appears to travel via the Na^+-K^+ pump, and little change is expected from an ouabain inhibition.

2. Cl^- Fluxes

Unidirectional Cl^- fluxes were also large (7.3-12.3 $\mu eq/hr \cdot cm^2$) though slightly less than that previously reported by Kishida, et al.[35] Though the small number of experiments lack statistical significance, the net Cl^- flux of 1.7 $\mu eq/hr \cdot cm^2$ in the blood-aqueous direction is consistent with that found by Kishida, et al.[35] and Kishida and Sasabe.[33] It is unlikely that this Cl^- flux represents an actual active Cl^- transport across the I-CB since (1) removal of Cl^- from the bathing solution has little influence on the SCC; and (2) furosemide had only a minor effect on SCC in this study. Ouabain is known to inhibit active Cl^- transport in epithelia containing a Na^+-Cl^- cotransport system. However, in 5 of 6 experiments, ouabain had no significant effect on the unidirectional Cl^- fluxes.

3. Comparison of Unidirectional Ionic Fluxes with Electrical Resistance

The mean value for electrical resistance from experiments

measuring Na^+ and Cl^- fluxes was about 120 $\Omega \cdot cm^2$, corresponding to an electrical conductance of 8.33 mS/cm^2. Krupin, et al.[26] had reported an electrical resistance of the I-CB to be 152 $\Omega \cdot cm^2$. Since only a small fraction of the unidirectional Na^+ and Cl^- fluxes represents a net flux (pump component), one should be able to assume that most of the Na^+ and Cl^- moves across the I-CB via the diffusional pathway. However, electrical conductance ($\Delta I/\Delta V$) does not agree with the conductance calculated from the partial conductance equation using the unidirectional Na^+ and Cl^- flux values. Possible contribution of other ions to the ionic conductance only increases the discrepancy. Thus, it is concluded that about 62% of the Na^+ and Cl^- fluxes traverse the membrane via non-diffusional pathways.

In conclusion, analysis of the flux determinations reveals large values for Na^+ and Cl^- unidirectional fluxes, variability between preparations, and a different net flux than expected from the SCC and drug effects. Thus, the usefulness of unidirectional fluxes to characterize I-CB transport mechanisms is questionable until more information is available about this preparation.

B. Effects of Pharmacological Agents

Figure VI below is the model taken from the manuscript to aid in interpreting the effects of various drugs on the SCC of the rabbit I-CB.

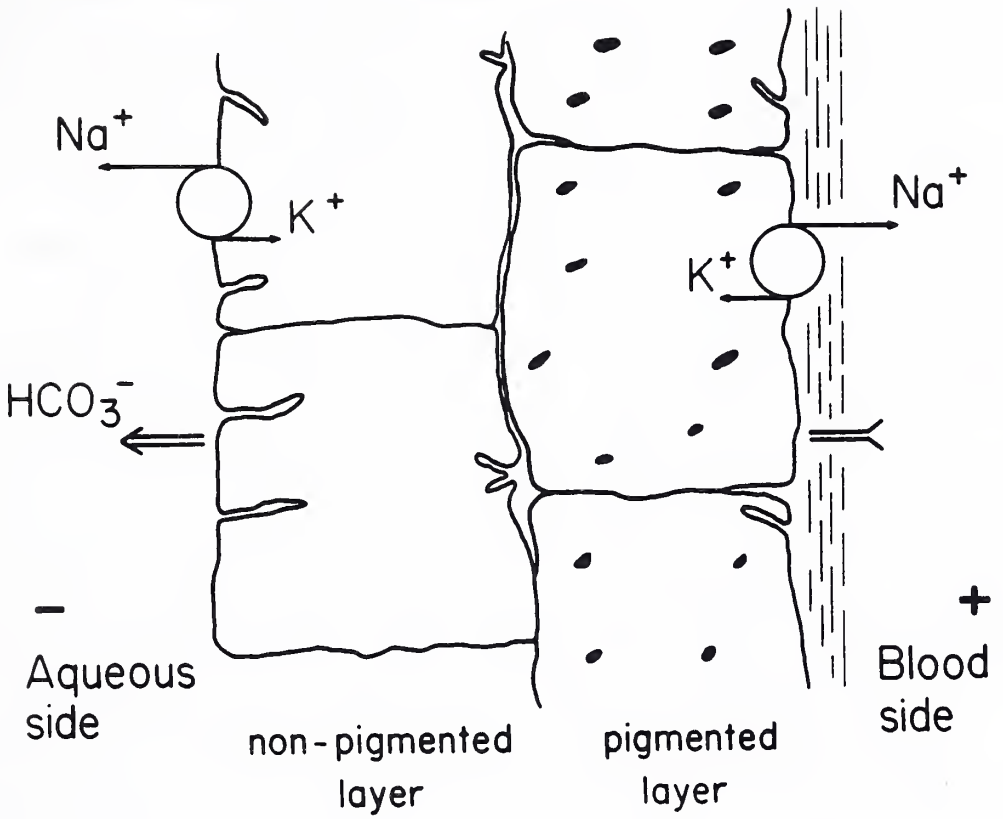

FIGURE VI. Schematic diagram of ionic transport across the isolated rabbit ciliary epithelium.

1. Ouabain

Aside from examining the effect of ouabain on unidirectional Na^+ and Cl^- fluxes, my experiments reproduced the previously reported effects of ouabain at $5x10^{-5}$ M concentration: (1) inhibition of the SCC when ouabain was on the blood side, and (2) initial stimulation of the SCC followed by inhibition of the SCC when ouabain was on the aqueous side. Krupin, et al.[26] had described this phenomenon as being due to a final common ouabain inhibitory effect on the blood side.

Na^+-K^+ pump. However, my experiments, having been carried out for longer duration, revealed that when ouabain is on the blood side, the inhibition of SCC is followed by an increase in SCC after about an hour. This indicates that ouabain on the blood side reaches the opposite aqueous side of the I-CB after about an hour and begins to inhibit the Na^+-K^+ pump on that side, too. Similarly, following ouabain's addition to the aqueous side, the SCC is stimulated for about an hour, after which permeation to the opposite blood side results in SCC diminution. Thus, ouabain's action is not via a specific final common inhibitory effect on the blood side Na^+-K^+ pump as suggested by Krupin, et al.[26] This is because ouabain's ability to diffuse or be transported bidirectionally through the I-CB enables it to inhibit the opposite-sided pump regardless of whether the initial ouabain effect was on the blood or aqueous side.

The effect of ouabain on the I-CB in HCO_3^- -free medium was also investigated. It was found that prior to ouabain's addition, the PD was low positive or zero. However, the absence of a PD due to the lack of HCO_3^- did not necessarily mean a non-viable preparation. Ouabain, in the absence of HCO_3^- , without any appreciable PD, exerted the same dual effect as in HCO_3^- -rich solution. There was no difference between the stimulation produced by ouabain on the aqueous

side in HCO_3^- -rich or HCO_3^- -free solution. However, the inhibitory effect of ouabain on the blood side in HCO_3^- -free solution was significantly smaller than in HCO_3^- -rich solution.

2. Amphotericin B

The dual effect of ouabain on the SCC in regular Tyrode's solution helps demonstrate the electrophysiological and metabolic integrity of the two cell layers of the I-CB. To further support this hypothesis, experiments using amphotericin B were done. Amphotericin B is known to stimulate active ionic transport by increasing the apical membrane permeability to ions and causing the pump on the basolateral membrane to be more efficient. If amphotericin B creates channels in parallel with the pump in one basolateral membrane, it should indirectly stimulate active transport by the pump in the opposite basolateral membrane.

Like ouabain, but by a different mechanism, amphotericin B on the aqueous side produced a large increase in SCC, presumably by stimulating the Na^+-K^+ pump in the blood layer. The amphotericin B stimulation of the SCC was usually much more considerable and of a longer duration than that of ouabain. Following stimulation by amphotericin B, ouabain added to the aqueous side had little or no effect, suggesting that the aqueous side Na^+-K^+ pump had been completely shunted. Ouabain addition to the blood side, following pre-treatment of the aqueous side with amphotericin B, resulted in an SCC inhibition that overcame most, if not all, of the previous amphotericin B stimulation.

The symmetrical experiments were carried out. Amphotericin B added to the blood side led to a moderate SCC inhibition, possibly by facilitating the transfer of Na^+ into the aqueous side solution. Subsequent addition of ouabain to the blood or aqueous side elicited a response similar to when ouabain was acting alone. This suggests that the increase in permeability was not large enough to completely shunt the Na^+-K^+ pump of the pigmented (blood) layer.

To confirm that the amphotericin B effects were related to Na^+-K^+ pump stimulation independent of HCO_3^- transport, the effect of amphotericin B on SCC was studied in HCO_3^- -free solution. The results were similar to those in HCO_3^- -rich solution. Thus, the changes in SCC by amphotericin B are due to stimulation of pumps not related to HCO_3^- transport.

Amphotericin B was also used to examine the possibility that Cl^- transport may be restricted under normal conditions but facilitated once the membrane permeability was increased by the polyene antibiotic. Nevertheless, I-CB's bathed in amphotericin B were only minimally affected by furosemide, as in control conditions.

To investigate whether the amphotericin B - stimulated SCC included a greater than usual stilbene-inhibitable fraction, the disulfonic stilbene, DIDS, was added to the aqueous side following SCC stimulation by amphotericin B on the aqueous side. DIDS inhibition of the SCC was the same as if amphotericin B had not been present. Furthermore, I-CB's pre-treated with DIDS on the aqueous side were stimulated by aqueous addition of amphotericin B as if DIDS

had not been previously present.

It is obvious that the above series of experiments shed significant light on I-CB transport mechanisms. Amphotericin B has proven to be a useful tool to partially dissect the transport properties of the two cell layers of the I-CB. It has significant potential to help further define the transport properties of one cell layer preferentially over the other.

3. Calcium Ionophore

Calcium ionophore A23187 in the cornea and other epithelia is known to increase cellular calcium concentration and mimic the effect of epinephrine, cAMP, and phosphodiesterase inhibitors in stimulating active Cl^- transport. However, A23187 caused no change in the electrical parameters of the I-CB. Since it had been previously shown to increase IOP of albino rabbits *in vivo*, it must do this via a mechanism other than stimulating aqueous humor secretion.

4. Epinephrine

Epinephrine had no effect on the aqueous side, suggesting the absence of receptors on the non-pigmented layer. It did, however, cause a small but consistent reduction in the SCC when added to the blood side. Thus, a direct effect of epinephrine on the I-CB requires entry into the circulation. Furthermore, the secretion-dependent effect of epinephrine on IOP may be minor when compared to that due to modification of outflow channels.

5. Phosphodiesterase Inhibitors

Theophylline and isobutyl-methylxanthine, both phospho-

diesterase inhibitors, stimulated the SCC. Note that such an effect does not necessarily mean an increase in aqueous humor flow. It could instead be the result of a reduction in the transport of Na^+ (or other positive ions) from blood to aqueous side.

6. Furosemide

Furosemide had previously been reported to inhibit the SCC of rabbit and toad isolated I-CB; and Cl^- transport has been described in rabbit, toad, and cat I-CB. However, in my experiments, furosemide had only a minor inhibitory effect on the SCC. Thus, the contribution of Cl^- transport to the SCC of the rabbit I-CB remains unclear.

7. Carbonic Anhydrase Inhibitor

The carbonic anhydrase inhibitor, trifluormethazolamide, caused an SCC inhibition. It had been previously shown to decrease IOP and aqueous humor secretion when added to rabbit eyes <u>in vivo</u>. Thus, assuming that the reduction in SCC is due to inhibition of HCO_3^- transport from blood-to-aqueous side, the effect of TFM is consistent with a reduction in aqueous humor secretion.

8. Disulfonic Stilbene

The effect of the disulfonic stilbene, DIDS, was investigated. While DIDS had no effect on the SCC when added to the blood side, there was a clear SCC inhibition by DIDS on the aqueous side. The effect of DIDS in HCO_3^- -free medium was significantly less than that in HCO_3^- -rich medium.

Disulfonic stilbenes are known to inhibit anion exchange systems in many cells. Thus, it is assumed that it acts on the I-CB by blocking either anion exchangers or anion-specific pathways in the membrane. HCO_3^- transport is the most likely anion system inhibited by DIDS, since (1) the involvement of Cl^- transport as a component of the SCC is dubious, and (2) DIDS had only a minimal effect in HCO_3^- - free medium.

VIII. CLOSING REMARKS

Transport properties in the isolated iris-ciliary body of the albino rabbit were investigated independent of such *in vivo* influences as systemic and local metabolic, humoral, circulatory, or neurologic factors. Only a few attempts have been successful in mounting the iris-ciliary body to demonstrate which ions are actively transported. Thus, the field of ciliary body electrophysiology has only just begun to be explored.

Our knowledge of the fundamental mechanisms behind aqueous humor secretion depends on continued study and perseverance. Treatment for glaucoma is aimed at diminishing aqueous humor production or at facilitating its outflow. To effect a cure for glaucoma, an understanding of the normal cellular processes regulating aqueous humor flow and how drugs act upon them is essential. To this end, experimental methods must continue to be used and improved to study the basic physiology and pharmacology of fluid movement of the eye.

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